Phosphorylation of p67^{phox} in the neutrophil occurs in the cytosol and is independent of p47^{phox}

Louisa V. Forbes[a,*], Stephen J. Moss[b], Anthony W. Segal[a]

[a] *Department of Medicine, University College London, 5 University Street, London WC1E 6JJ, UK*
[b] *Medical Research Council Laboratory for Molecular Cell Biology, University College London, London WC1E 6BT, UK*

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Abstract p67^{phox} and p47^{phox} are phosphorylated in the course of stimulation of the NADPH oxidase in neutrophils. Isolated neutrophil cytosol can phosphorylate both of these proteins in vitro. Phosphoamino acid analysis showed that isolated membranes can tyrosine-phosphorylate p67^{phox} in vitro. Further experiments with anti-phosphotyrosine antibodies did not support a role for tyrosine phosphorylation of p67^{phox} in the cell. A phosphopeptide analysis showed that the phosphorylation of p67^{phox} is unchanged in the absence of p47^{phox}. These results further characterise the phosphorylation of p67^{phox} and provide evidence that this is a cytosolic event independent of interaction with p47^{phox} and the membrane.

Key words: Nicotinamide adenine dinucleotide phosphate, reduced form oxidase; Phosphorylation; Phosphopeptide mapping

1. Introduction

p67^{phox} is a component of the nicotinamide adenine dinucleotide phosphate, reduced form (NADPH) oxidase, the multicomponent enzyme that causes the production of superoxide in phagocytes. It is one of the components that is located predominantly in the cytosol and a small proportion translocates to the membrane upon activation of the neutrophil. For p67^{phox}, p47^{phox}, p40^{phox} and Rac, the translocation has been estimated in the range of 2–20% of each protein [1]. The higher values averaging 10–15% occur with stimulation by phorbol ester, whereas only 2–5% translocates with receptor-mediated stimulation [2,3]. The formation of an active NADPH oxidase at the membrane is dependent on these components, as evident by flavocytochrome-positive autosomal chronic granulomatous disease (CGD), which is caused by a deficiency of either p67^{phox} or p47^{phox}.

In addition to translocation, the phox proteins become phosphorylated upon stimulation of the NADPH oxidase. There is growing proof that phosphorylation plays an essential role in the activation process. In addition to temporal correlation between the phosphorylation events and the activation of the NADPH oxidase [4–6], the requirement of phosphorylation sites in p47^{phox} is supported by the effect of mutagenesis of p47^{phox} on the translocation and activity in transfected cell systems [7–9]. The phosphorylation of several residues in p47^{phox} takes place in the cytosol [10] and more phosphorylation occurs after translocation to the flavocytochrome at the membrane [11,12]. It is believed that phosphorylation confers activation by causing conformational changes which lead to certain interactions between the components. Experimental evidence for this comes from spectroscopic measurements of alterations in the p47^{phox} conformation [13,14], protein-protein interaction studies [15,16] and the phosphorylation-dependent activation of cell-free oxidase systems [13,17].

Unlike the phosphorylation of p47^{phox} which was the first biochemical abnormality identified in autosomal CGD [18,19], the phosphorylation of p67^{phox} has been less intensively investigated. We have recently reported that the major site of phosphorylation is the single amino acid threonine-233 [20], which contrasts with the multiple site phosphorylation of the C-terminus of p47^{phox} [21]. The phosphorylation of threonine-233 can be brought about in vitro by isolated cytosol or mitogen-activated protein kinase [20]. However, as with the other phox components, the exact dynamics of the events of phosphorylation, intercomponent interactions and translocation to the membrane remain to be deciphered for p67^{phox}. Here, we further investigate the phosphorylation of p67^{phox}, finding evidence for the reaction taking place in the cytosol by a mechanism independent of any interaction with p47^{phox} or the membrane.

2. Materials and methods

2.1. Cell preparation and fractionation

Neutrophils were prepared [22] from buffy coats or freshly drawn heparinised blood, from healthy volunteers. The media used when labelling neutrophils was phosphate-free HEPES-buffered saline (HBS) (20 mM HEPES, 137 mM NaCl, 2.7 mM KCl, 1 mM $MgSO_4$, 1 mM $CaCl_2$, pH 7.4). Neutrophils were resuspended at 5×10^7/ml in HBS/glucose/bovine serum albumin (0.025%(w/v)) and incubated with $^{32}P_i$ at 0.1–0.5 mCi/ml, 30°C, 60 min. For the study of CGD cells, EBV-transformed B-cells from normal subjects and p47^{phox} deficient patients were grown in culture and incubated at 1×10^7/ml in phosphate-free RPMI with 10%(v/v) dialysed foetal bovine serum and $^{32}P_i$ at 0.1–0.5 mCi/ml, 37°C, 4 h. Stimulation of cells by serum-opsonised zymosan (SOZ, 1 mg/ml, 7 min) or 12-phorbol myristate 13-acetate (PMA, 1 μg/ml, 5 min) or *N*-formyl-methionyl-leucyl-phenylalanine (fMLP, 1 μM, 1 min) following a pre-incubation of the cells with cytochalasin B (5 μg/ml, 5 min) was quenched by the addition of a more than five times volume excess of ice-cold media. Cells were sedimented by centrifugation at $200\times g$, 4°C, 3 min, then lysed by sonication in relaxation buffer (10 mM Pipes, 3 mM NaCl, 100 mM KCl, 3.5 mM $MgCl_2$, 1.25 mM EGTA, pH 7.3). Protease and phosphatase inhibitors were present during sonication and subsequent procedures (1 mM diisopropyl fluorophosphate, 0.2 mM phenylmethylsulphonyl fluoride, 10 μg/ml *N*-α-*p*-tosyl-L-lysine chloromethyl ketone, 10 μg/ml leupeptin, 10 μg/ml pepstatin, 1 mM benzamidine, 20 mM NaF, 5 mM sodium pyrophosphate, 200 μM $NaVO_4$, 100 nM microcystin). Cytosol was prepared by centrifuging the lysate at $500\times g$,

*Corresponding author. Fax: (44) (171) 209 6211.
E-mail: l.forbes@ucl.ac.uk

Abbreviations: CGD, chronic granulomatous disease; EBV, Epstein-Barr virus; NADPH, nicotinamide adenine dinucleotide phosphate, reduced form

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4°C, 5 min, followed by 100 000×*g*, 4°C, 12 min. Membranes were prepared by centrifuging the cell lysate at 150 000×*g*, 4°C, 30 min, through a discontinuous sucrose gradient and collecting the interface between layers of 15% and 34% (w/v) sucrose.

2.2. Immunoprecipitation experiments

Immunoprecipitations were carried out in solubilisation buffer (20 mM Tris, 150 mM NaCl, 1% (v/v) Triton X-100, 1% (w/v) sodium deoxycholate, 0.1% (w/v) SDS, pH 7.3), as published previously [20]. Briefly, the cellular fraction was pre-adsorbed against protein-A sepharose to reduce the non-specific binding and then incubated with an affinity-purified antibody raised in rabbits against either full-length GST-p67^{phox} or GST-p47^{phox} fusion protein. Immunocomplexes were sedimented after incubation with protein-A sepharose. After washing, the immunoprecipitate was boiled in Laemmli sample buffer and subjected to SDS-PAGE. The bands from Coomassie blue staining and autoradiography were quantified using a Fuji BAS1000 PhosphorImager or an Alpha Innotech AlphaImager 2000.

2.3. In vitro phosphorylation

25 μl reactions were set up on ice consisting of 5 μl 5×kinase buffer (50 mM $MgCl_2$, 2.5 mM $CaCl_2$, 100 mM HEPES, pH 7.4), 5 μl 0.5 mM ATP at approximately 1000 cpm/pmol, 0.5–5 μg recombinant p67^{phox} or p47^{phox} protein) and kinase in the form of cytosol ($1–5\times10^4$ cell equivalent (eq)) or membranes ($1–3\times10^6$ cell eq). Following an incubation of 30°C, 20 min, the reactions were stopped by the addition of 3 μl 10×Laemmli sample buffer, then boiled and subjected to SDS-PAGE.

2.4. Phosphopeptide mapping

Phosphorylated p67^{phox}, either from an immunoprecipitate or an in vitro phosphorylation reaction, was excised from a SDS-PAGE gel and subjected to tryptic digestion as previously described [20]. A CBS Scientific Hunter Thin Layer Electrophoresis 7000 system was used for 2D phosphopeptide mapping and images were obtained with a Fuji BAS 1000 PhosphorImager. The first dimension of resolution was by electrophoresis in pH 1.9 buffer (2.5% (v/v) formic acid, 7.8% (v/v) acetic acid), 1000 V, 35 min and the second dimension was by thin layer chromatography in Phospho-chromatography buffer (7.5% (v/v) acetic acid, 25% (v/v) pyridine, 37.5% (v/v) butanol), 6–8 h. For phosphoamino acid analysis, consecutive electrophoresis steps of 1500 V, 20 min, pH 1.9 buffer and 1300 V, 16 min, pH 3.5 buffer (0.5% (v/v) pyridine, 5% (v/v) acetic acid) were carried out. Phosphoamino acid markers were detected by ninhydrin spray (0.25%(w/v) in acetone) and their positions were outlined on the phosphorimage.

2.5. Phosphotyrosine immunoblotting

SDS-PAGE gels were blotted onto nitrocellulose using a LKB 2117 Multiphor semidry electroblotter. The anti-phosphotyrosine antibody was the recombinant RC20:HRPO from Transduction Laboratories. This antibody is conjugated with horse radish peroxidase eliminating secondary antibody incubations. Incubations were carried out following the manufacturer's instructions, including using BSA blocking solutions without milk powder and detection by the enhanced chemiluminescence method.

3. Results and discussion

Previous studies have established that phosphorylated p67^{phox} and p47^{phox} are present in the cytosol of neutrophils after stimulation of the NADPH oxidase [5,20,23–25]. In order to directly compare the increase in the phosphorylation of p67^{phox} upon stimulation of neutrophils with that of p47^{phox}, immunoprecipitations of p67^{phox} and p47^{phox} were carried out in parallel on cytosol from ^{32}P-radiolabelled cells. The relative phosphorylation was quantified by measuring the ratio of the immunoprecipitated radioactivity to the amount of immunoprecipitated protein for p67^{phox} and p47^{phox} separately (Fig. 1). Some phosphorylation of both p67^{phox} and p47^{phox} was evident in the neutrophils which were not exposed to an agonist of the NADPH oxidase ('unstimulated'). This could indicate either a physiological basal level of phosphorylation in

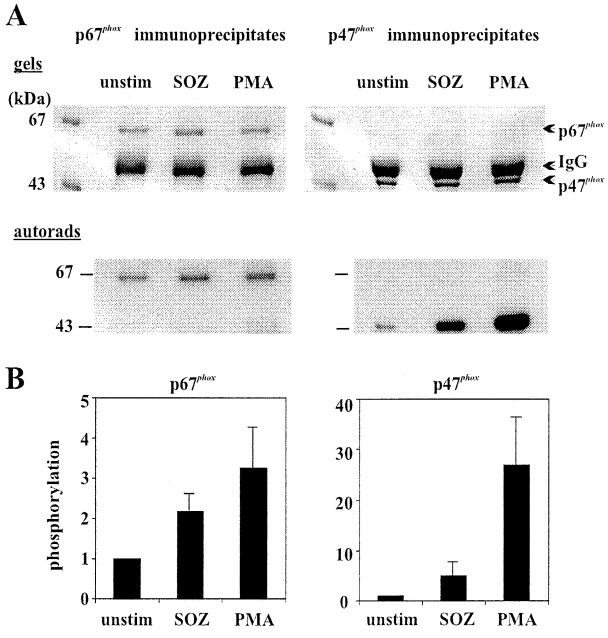

Fig. 1. Comparison of the p67^{phox} and p47^{phox} phosphorylation in neutrophils. (A) Immunoprecipitates of p67^{phox} or p47^{phox} from 4.5×10^7 cell eq cytosol of radiolabelled neutrophils were subjected to SDS-PAGE, then Coomassie-stained (gels) and exposed to film for 2 weeks (autorads). Neutrophils were either unstimulated or stimulated with 1 mg/ml SOZ for 5 min or 1 μg/ml PMA for 6 min, prior to lysis and immunoprecipitation. (B) Quantitation of the increase in the phosphorylation upon activation relative to unstimulated cells, from four experiments, error bars indicate S.D. Values were derived from band densitometry of the autorad signal and were corrected for the amount of protein detected by Coomassie staining.

resting neutrophils or that the cells received some stimulation as a result of handling during the experiment. An increase in phosphorylation was observed in the conditions of NADPH oxidase activation for both proteins, but was much higher for p47^{phox} than for p67^{phox}. This is consistent with there being one major phosphorylation site in p67^{phox} [20] and multiple sites in p47^{phox} [21].

Upon incubation of the cells with SOZ, the level of phosphorylation increased by a factor of 2.17 ± 0.52 and 4.97 ± 2.50 for p67^{phox} and p47^{phox}, respectively. The magnitude of phosphorylation was significantly higher using the phorbol ester agonist, PMA, with increases of 3.24 ± 1.14 and 26.77 ± 10.12 for p67^{phox} and p47^{phox}, respectively. As a particulate agonist that causes phagocytosis, SOZ is likely to represent a more physiological stimulation of the NADPH oxidase in the neutrophil than PMA. The phosphorylation state of p67^{phox} has been shown to be the same by SOZ and PMA stimulation [20], therefore the higher phosphorylation by PMA must represent more molecules of phosphorylated p67^{phox}. The phosphorylation sites of p47^{phox} by particulate stimulation have not been mapped. The disproportionate increase in the phosphorylation of p47^{phox} by PMA stimulation, approximately eight times that of SOZ stimulation, is likely to be caused by the direct activation of PKC for which there are several candidate sites in p47^{phox} [26].

In vitro phosphorylation of recombinant p67^{phox} and p47^{phox} can be achieved using cytosol isolated from neutrophils. Not surprisingly, p47^{phox} becomes more heavily phosphorylated than p67^{phox} (Fig. 2). However, the difference in

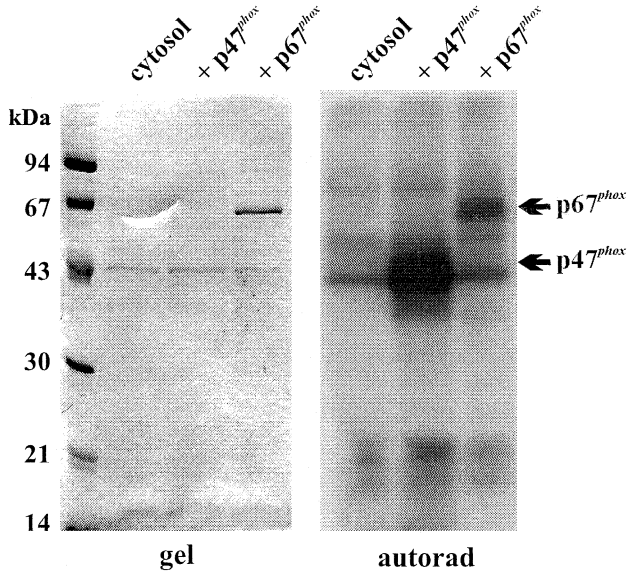

Fig. 2. Comparison of the in vitro phosphorylation of p67^{phox} and p47^{phox} by neutrophil cytosol. A Coomassie-stained gel and autoradiograph of the in vitro phosphorylation reactions of recombinant p67^{phox} and p47^{phox} by unstimulated neutrophil cytosol. Background phosphorylation is shown as cytosol alone.

the magnitude of phosphorylation cannot be accounted for solely by the higher number of phosphorylation sites in p47^{phox}. That is, there is a greater difference between the phosphorylation of p67^{phox} and p47^{phox} in the in vitro experiment than that observed in the immunoprecipitations. This suggested to us that p67^{phox} may require a co-factor to achieve the maximal phosphorylation. The addition of either the small G-protein Rac or p40^{phox} did not enhance the phosphorylation of p67^{phox} in vitro. Neither did the activation state of the cytosol have an effect, as the same level of phosphorylation was achieved using cytosol isolated from unstimulated and stimulated neutrophils (LV Forbes, data not shown).

To investigate whether the phosphorylation of p67^{phox} could involve an interaction with the membrane, the cytosol in the in vitro experiment was replaced by the membranes

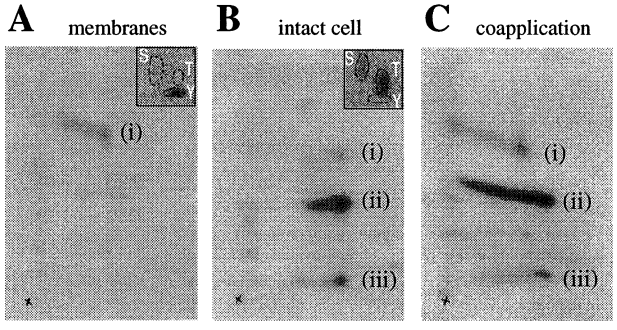

Fig. 3. Phosphopeptide analysis of the isolated membrane and intact cell phosphorylation of p67^{phox}. 2-dimensional phosphopeptide maps of (A) the in vitro phosphorylation of recombinant p67^{phox} by unstimulated neutrophil membranes, (B) neutrophil p67^{phox}, immunoprecipitated from the cytosol of PMA-stimulated cells and (C) co-application of duplicate samples as applied to A and B. Insets are phosphoamino acid analyses, indicating the resolution of phosphoserine (S), phosphothreonine (T) and phosphotyrosine (Y). Phosphopeptide positions (i, ii, iii) are discussed in the text.

fraction of the neutrophil lysate. Phosphoamino acid analysis of membrane-phosphorylated p67^{phox} showed that phosphorylation occurred on tyrosine residues (Fig. 3A), in contrast to the threonine and serine phosphorylation observed in immunoprecipitated p67^{phox} (Fig. 3B). The immunoprecipitations were carried out from neutrophil cytosol because the phosphorylation signal of the small amounts of p67^{phox} at the membrane is not intense enough to map by these methods. We have previously shown that the strongest phosphopeptide signal from cytosol-immunoprecipitated p67^{phox} (signal (ii)) is due to the phosphorylation of threonine-233 [20]. The phosphopeptide maps of p67^{phox} confirmed that the phosphorylation by membranes was on a different site from threonine-233, but that it may occur weakly in the intact cell (Fig. 3B and C, signal (i)). Phosphotyrosine is known to be relatively labile in the acid hydrolysis of a two-dimensional phosphoamino acid analysis, making it harder to detect than phosphothreonine or phosphoserine [27]. Therefore, this result may indicate that only by in vitro phosphorylation with membranes was the stoichiometry of the tyrosine phosphorylation sufficient for detection by acid hydrolysis. It was found that a low tyrosine phosphorylation could be detected in the p67^{phox} immunoprecipitated from neutrophils using sensitive anti-phosphotyrosine antibodies.

Anti-phosphotyrosine antibodies were used to address the issue of whether tyrosine phosphorylation of p67^{phox} is associated with the NADPH oxidase activity. Tyrosine phospho-

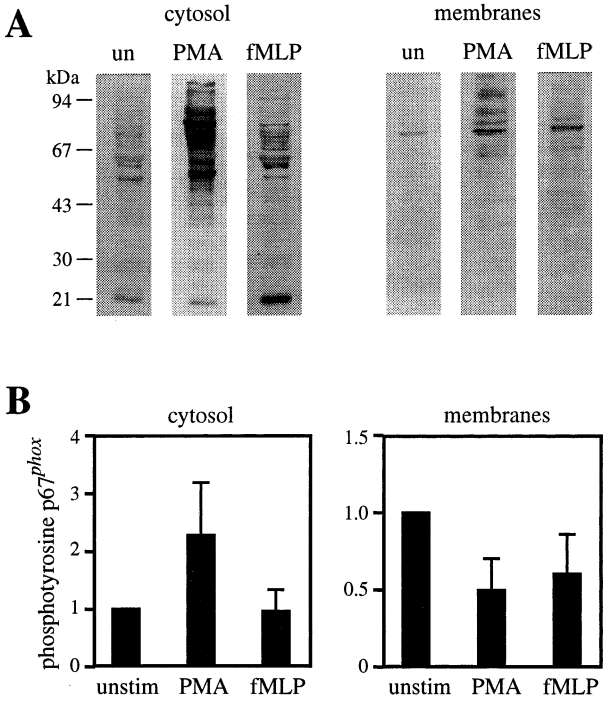

Fig. 4. Phosphotyrosine analysis of p67^{phox}, immunoprecipitated from neutrophils. (A) Anti-phosphotyrosine immunoblots of 7.5×10^5 cell eq cytosol and 4.5×10^7 cell eq membranes from unstimulated, PMA- or fMLP-stimulated neutrophils. The blots were developed by the ECL method. (B) Quantitation of the amount of tyrosine phosphorylation by densitometric analysis of immunoblots of p67^{phox} immunoprecipitates from 7.5×10^7 cell eq cytosol and 7.5×10^8 cell eq membranes. The ratio of the phosphotyrosine signal to the p67^{phox} signal is normalised against that of unstimulated cells which was assigned 1.00. The entire experiment was performed three times, the error bars indicate S.D.

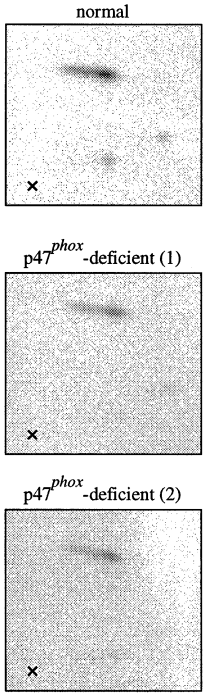

Fig. 5. Phosphorylation of p67^{phox} in p47^{phox} deficient EBV B-cells. 2-dimensional phosphopeptide maps of p67^{phox}, immunoprecipitated from radiolabelled and PMA-stimulated EBV B-cells, from a normal subject and two p47^{phox} deficient CGD patients.

rylation of multiple proteins is known to occur upon stimulation of the NADPH oxidase [28,29]. Fig. 4A confirms that this takes place in the cytosol and at the membranes. p67^{phox} was immunoprecipitated from cytosol and membrane fractions of neutrophils and the phosphotyrosine signals were quantified by densitometry of immunoblots (Fig. 4B). The extent of tyrosine phosphorylation was normalised for the amount of p67^{phox} protein detected by subsequent blotting against anti-p67^{phox} antibody for the cytosol and membranes separately. A very weak tyrosine phosphorylation was detected in p67^{phox} of unstimulated cells, with the membrane p67^{phox} showing a stronger phosphotyrosine signal than cytosolic p67^{phox}. This would be consistent with the tyrosine phosphorylation occurring mostly at the membrane.

The observed increase in tyrosine phosphorylation of cytosolic p67^{phox} upon stimulation of neutrophils with PMA, but not by the membrane receptor-mediated agonist fMLP, is probably indicative of PMA's documented inhibition of tyrosine phosphatases [30] rather than up-regulation of a physiologically active tyrosine kinase. Immunoprecipitation of membrane-localised p67^{phox} showed a decrease in the extent of tyrosine phosphorylation by approximately half, despite an increase in the amount of p67^{phox} at the membrane. Therefore, the fraction of cytosolic p67^{phox} that translocates to the membrane upon stimulation is not tyrosine-phosphorylated, suggesting that tyrosine phosphorylation is not a requirement for the membrane localisation. As the strongest phosphorylation signal was from the small amount of p67^{phox} at the membrane in unstimulated cells, these results do not support a role for the p67^{phox} tyrosine phosphorylation in the NADPH oxidase activation.

We investigated whether a role for p47^{phox} was implicated in the phosphorylation of p67^{phox}. Interaction between p67^{phox} and p47^{phox} in the cytosol has been suggested as a control mechanism in the activation of the NADPH oxidase [16]. It has previously been reported that p47^{phox} phosphorylation is normal in the absence of p67^{phox} [12,24,31,32], suggesting that phosphorylation does not require an intermolecular complex in the cytosol. We investigated whether the phosphorylation of p67^{phox} is similarly independent of p47^{phox}. We have previously shown that the p67^{phox} phosphorylation in B lymphocytes is virtually the same as in neutrophils, with only small differences in the minor phosphopeptide signals [20]. EBV-transformed B-cell lines were used to compare normal cells with p47^{phox} deficient cells. The phosphopeptide map of p67^{phox}, that was immunoprecipitated from normal cells, was the same as that from two p47^{phox} deficient CGD patients (Fig. 5). The B-cell lines were proven to be p47^{phox} deficient by immunoblotting the whole cytosol with an affinity-purified antibody against p47^{phox}. Hence, the phosphorylation of p67^{phox} appears to be independent of p47^{phox}.

In summary, the phosphorylation of p67^{phox} is a more subtle event than the multiple site phosphorylation of p47^{phox}. Although tyrosine phosphorylation of p67^{phox} may occur weakly in the cell, the predominant phosphorylation that correlates with activation of the NADPH oxidase is due to serine/threonine phosphorylation. This phosphorylation is unaffected by p47^{phox} and is cytosol-based rather than occurring at the membrane. These data support the hypothesis that phosphorylation of p67^{phox} occurs in the cytosol, followed by translocation to the membrane, where p67^{phox} assembles with p47^{phox} and the flavocytochrome-based NADPH oxidase complex.

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